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ORIGINAL PAPER

Cloning of vacuolar H^+-ATPase subunit c genes from Japanese iris, and functional characterization in yeast

ZHOU Ai-min • WU Duo • CHE Dai-di • LIU Shen-kui • YANG Chuan-ping • WANG Jin-gang

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Abstract: Five different isoforms (*IrlVHA-c1–c5*) of V-ATPase subunit c (*VHA-c*) were cloned from a Japanese iris (*Iris lactea* Pall. var. chinensis Fisch. Koidz) cDNA library using degenerate primers PCR and the 5'-RACE technique. The sequence analysis showed the open reading frame (ORF) of the *IrlVHA-c1–c5* to be 495 bp, corresponding to a protein of 164 amino acids. Among the five isoforms, *IrlVHA-c1* and *IrlVHA-c2* are completely homologous. The *IrlVHA-c* protein is localized at the vacuolar membrane as indicated by a green fluorescent protein (GFP) marker. Its over-expression in yeast could enhance yeast tolerance to NaCl stress. These results show that there are at least five genes encoding different isoforms of *IrlVHA-c* in Japanese iris and *IrlVHA-c* is important for the function of V-ATPase.

Keywords: green fluorescent protein; Japanese iris; subunit c; V-ATPase; yeast

Introduction

V-ATPase is an electrogenic proton pump, which is localized at endomembranes of eukaryotic cells. Protons are transported across intracellular membranes at the expense of energy consumption from ATP by V-ATPase. Thus vacuole, endoplasmic reticulum, Golgi, and lysosomes have proton motive force on their membranes. In plant cells, V-ATPase is important for the maintenance of intracellular pH and ion homeostasis, cell growth, and vesicle trafficking, as well as adaptation to environmental stresses such as salinity, drought, and heavy metals (Ratajczak 2000). V-ATPase is composed of a hydrophilic V_1-domain localized in the cytoplasm and a hydrophobic membrane-integral V_0-domain. Genomic sequences for 13 subunits of the multimeic V-ATPase complex have been identified in the genome of *Arabidopsis thaliana*: *VHA-A* to *VHA-H*, *VHA-a*, *VHA-c*, *VHA-d*, and *VHA-e* (Dietz et al. 2001; Sze et al. 2002).

Mostly, the subunit composition of V-ATPase resembles that of F-type ATPase. Subunit homology is the most significant for the large subunits A and B and for the proteolipid subunit c (Hilario and Gogarten 1998). Subunit c of the V_0 complex has been widely studied, and its genes from various fungal, plant, arthropod, mammalian and other vertebrates have been cloned. The 16 kDa proteolipid is one of the most conserved membrane proteins, with more than 65% identity among virtually all species. Subunit c is encoded by the *VMA3* gene in *Saccharomyces cerevisiae* and its interruption results in unstable V_0 complex and failure in assembling V_1, loss of V-ATPase activity, and associated dysfunction. This suggests it is a major component for the function of proton translocation by V-ATPase (Umemoto et al. 1990; Finbow and Harrison 1997). The protein contains four hydrophobic segments that cover two-thirds of all residues and make the protein highly hydrophobic but soluble in organic solvents (Finbow and Harrison 1997). The hydrophobic regions are interspersed with short extramembranous hydrophilic loops, while the loops between helices 1/2 and 3/4 are cytoplasmically exposed. The core of the V_0 complex is a hexamer of the subunit c protomers symmetrically arranged. This pore is 15-20 Å in diameter, larger than expected for proton translocation, but not unusual for a gap-junction channel characterized by permeability. It seems that, in V-ATPase, this pore is occluded, perhaps by the central 'spindle' subunit D (Finbow and Harrison 1997).

Finbow and Harrison (1997) hypothesized multiple genes en-

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ZHOU Ai-min • WU Duo • CHE Dai-di • WANG Jin-gang (✉)
Horticulture college, Northeast Agricultural University, Harbin, 150030, China. E-mail: wangjingang99@yahoo.com.cn

LIU Shen-kui
Alkali Soil Natural Environmental Science Center (ASNESC), Northeast Forestry University, Harbin 150040 China

YANG Chuan-ping • WANG Jin-gang
Key Laboratory of Forest Tree Genetic Improvement and Biotechnology, Ministry of Education, Northeast Forestry University, Harbin, 150040, China

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coding different isoforms of the multifunctional property of subunit c. Subunit c genes from several plants have already been cloned and their translated protein sequences revealed polypeptides with relative molecular masses between 16.6 and 16.7 kDa (Finbow and Harrison 1997). Subunit c is thought to carry protons and the rotation of a ring of six subunits c is considered essential to drive proton transport (Hirata et al. 2003). The 16 kDa integral protein or subunit c is encoded by the largest multigene family with five members in *A. thaliana* (Sze et al. 2002). Subunit c was the first multigene family reported in eukaryote V-ATPases (Sze et al. 1992; Perera et al. 1995), and this feature is conserved in other plants, including cotton (*Gossypium hirsutum*) (Hasenfratz et al. 1995), rice (*Oryza sativa*; Rice Genome Databases), and *Pennisetum glaucum* (Tyagi et al. 2006). Encoded by five homologs, genes of V-ATPase subunit c from *A. thaliana* (*AVAP1-AVAP5*) have identities between them varying from 87%–91% at the DNA level and 99.4%–100% at the protein level (Perera et al. 1995). Three different isoforms of vacuolar ATPase subunit c (*VHA-c*) from different plant tissues of *P. glaucum* have been cloned. Interestingly, environmental stress induced different levels of their expressions (Tyagi et al. 2006). In general, subunit c plays an important role in assembling the V-ATPase complex and in regulating its activity (Umemoto et al. 1990; Tyagi et al. 2005). Here, we studied IrlVHA-c to get a better understanding of its functions.

Materials and methods

Material

Iris lactea, provided by the Northeast Agricultural University of China, was grown in the field under normal conditions. Bacteria, yeast strains INVScI, and yeast transformants were selected on SD-Uracil medium (Nelson and Klinsky 1996). Plasmids pYES2 and pEGFP were selected and propagated in *Escherichia coli* strain JM109.

RNA isolation and RT-PCR

Total RNA from Japanese iris was isolated using the TRIZOL reagent (Invitrogen). Approximately 500 μg of total RNA or 5 μg of mRNA was used to synthesize first-strand cDNA using the cDNA Synthesis Reagent Kits (TaKaRa) according to manufacturer instructions. Using the tBLASTn program (http://www.ncbi.nlm.nih.gov/BLAST/) with the probe of *A. thaliana AVAP5* (GenBank No. NP179244), five candidates (GenBank No. XP002279080; XP002310846; XP002510486; NP001047092; NP001149195) were obtained. According to a conservative amino acid sequence, we designed degenerate primers VHA-F (ACNGC BCCCTTCTTCGGCTT) and VHA-R (CTAATCNGCTCKGGAYTGICC) to obtain a product of 471 bp using cDNA of *Iris lactea* as a template. We sequenced the product and designed specific primers 5′-RACE-R (5′-CGAGG AAGCCGAAGAAGGGAGCTGT-3′). Together with a 5′-Full RACE Kit (TaKaRa) and an inner primer 5′-RACE–F (5′-CGCG GATCCACAGCCTACTGATGATCAGTCGATG-3′), we got the full-length gene. Full length of the clone was 598 bp including a 103-bp 5′ untranslated region (UTR) and a 495-bp open reading frame (ORF). Specific primers IrlVHA-F (5′-ATGCCGTCCTTC AGCGGCGAC-3′) and IrlVHA-R (5′-CTAATCGGCTCTGGAT CGGC-3′) were used to obtain the five isoform genes.

Plasmid construction and yeast transformation

The ORF of *IrlVHA-c2–c5* was amplified from *IrlVHA-c* cDNA with the primers YES2-F (5′-GGATCCATGCCGTCCTT CAG-3′) and YES2-R (5′-TCTAGACTAATCGGCTCTGG-3′). In *IrlVHA-c2–c5* genes 5′ and 3′ were added to contain BamHI and XbaI restriction sites. All amplified fragments were sub-cloned into the pYES2 vector (Invitrogen) and the constructed vectors were introduced into yeast INVScI using the LiAc/PEG method (Gietz et al. 1995). For construction of GFP fusion proteins, the *IrlVHA-c2–c5* gene without the stop codon was amplified with the primers GFP-F (5′-GGATCCATGCCGT CCTTCAG-3′) and GFP-R (5′-GGTACCTAATCGGCTCTGG-3′), and then cloned into pEGFP vector by BamHI and KpnI sites. The GFP-IrlVHA-c fusion proteins were cloned into pYES2 vectors by BamHI and XbaI sites, respectively. Each plasmid was then digested and cloned into the corresponding sites of pYES2-GFP vectors to obtain the plasmids pYES2-GFP–IrlVHA-c2, pYES2-GFP-IrlVHA-c3, pYES2-GFP-IrlVHA-c4, and pYES2-GFP-IrlVHA-c5. These four GFP fusion plasmids and pYES2-GFP were introduced into yeast strain INVScI. The yeast transformants were cultured in liquid SC-Uracil medium with galactose for 24 h at 30°C and yeast cells were incubated with 0.02 mmol/L FM4-64 dye (Molecular Probes) for 30 min at 30°C. These cells were washed three times before confocal analysis, which was performed with a TCS SP2 laser-scanning confocal imaging system (Leica). GFP and FM4-64 fluorescent signals were detected with excitations at 488 nm and 543 nm, respectively. The yeast transformant of pYES2-GFP was used as a control.

Results

Sequence analysis of *IrlVHA-c* gene

The sequence analysis showed that the ORF of *IrlVHA-c1–c5* had 495 bp encoding a protein of 164 amino acids, which we predicted to have a molecular mass of 16.6 kDa and four transmembrane domains. In addition, *IrlVHA-c1* had a 103-bp 5'-UTR. The isoforms *IrlVHA*-c2–c5 were highly homologous to *IrlVHA*-c1 with 90.5%, 90.3%, 89.9%, and 89.9% identities at DNA level (Fig. 1), and with 96.3–100% identities at protein level. Therefore, *IrlVHA-c1* and *IrlVHA-c2* are homologs. GenBank Numbers for *IrlVHA-c1–c5* was GU001680, GU001681, GU001682, GU001683, and GU001684.

Proteins encoded by *IrlVHA-c1–c5* showed 95.1%–97.6% similarity with *AVAP5* of *A. thaliana* (GenBank No. NP179244), 94.5%–97.0% similarity with *VHA-c* from *Oryza sativa* (Gen-

Bank No. NP001047092), and 95.1%–97.6% similarity with *VHA-c* from *Sorghum bicolor* (GenBank No. XP002441892) (Fig. 2).

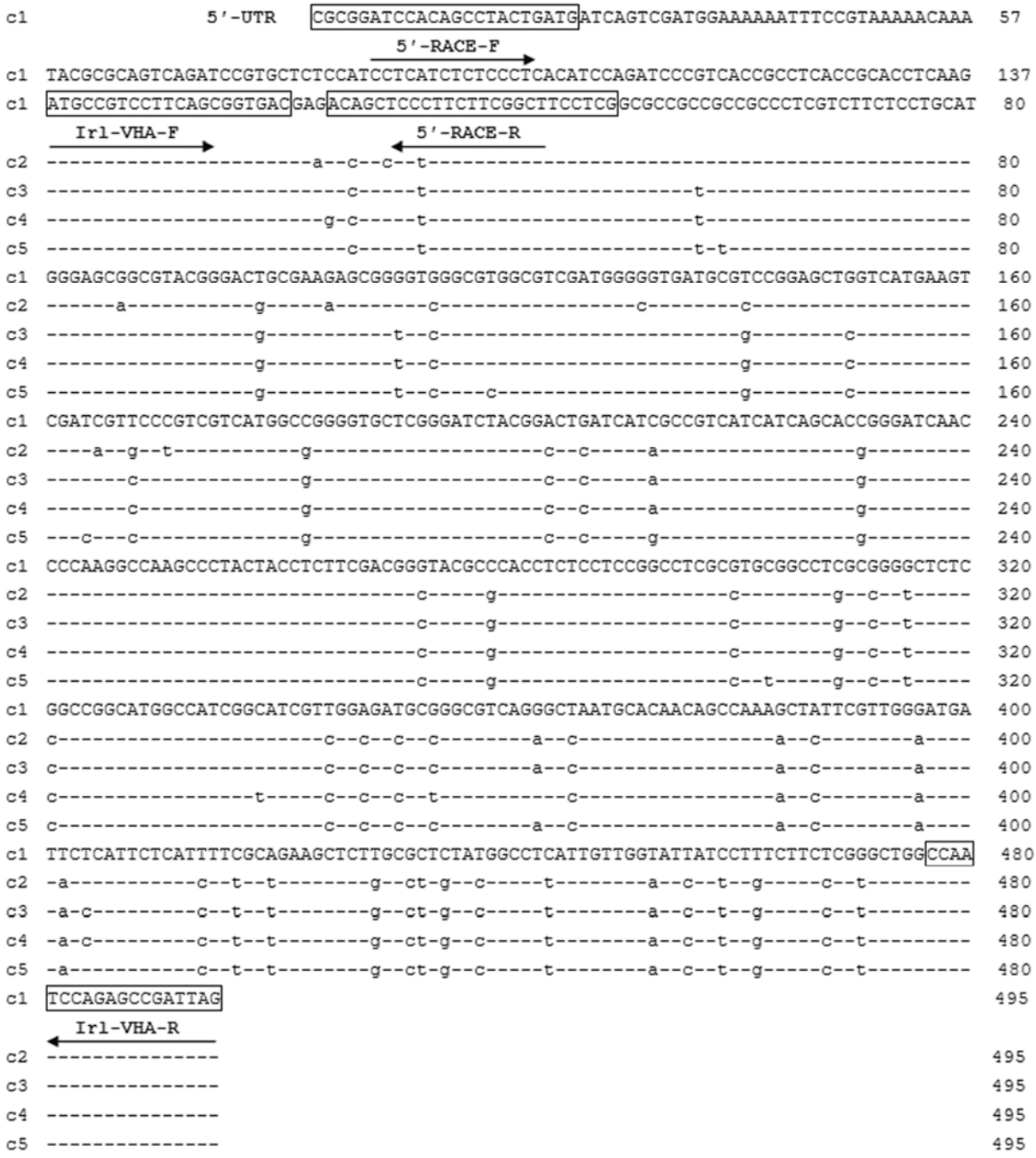

Fig. 1 Multiple alignment of nucleic acid sequences of *IrlVHA-c1*–*c5*. *IrlVHA-c1* (GU001680); *IrlVHA-c2* (GU001681); *IrlVHA-c3* (GU001682); *IrlVHA-c4* (GU001683); *IrlVHA-c5* (GU001684). Horizontal bars indicate identical nucleic acid residues. The c1 front-end 137 nucleic acid residues are 5′-UTR. The primers used for PCR and 5′-RACE are indicated.

In a phylogenetic analysis, *IrlVHA-c* and the cloned *VHA-c* homolog showed similarity greater than 94%, indicating that *VHA-c* is highly conserved (Drory and Nelson 2006). The *IrlVHA-c* proteolipid is a hydrophobic peptide and spans the membrane four times, as indicated by a hydropathy plot. This is a common feature of proteolipid subunit c of V-ATPases in all organisms. The glutamate residue in the 4th transmembrane helix is also conserved in the *IrlVHA-c* proteolipid (Fig. 3). This glutamate residue acts as a proton acceptor in the proton channel of the V_0 complex. N,N'-dicyclohexylcarbodiimide (DCCD) can bind to this glutamate residue and abolishes V-ATPase activity in all organisms so far examined (Stevens and Forgac1997; Nelson

and Harvey 1999). These results suggest that the cloned proteolipid subunit acts as a component of the V-ATPase.

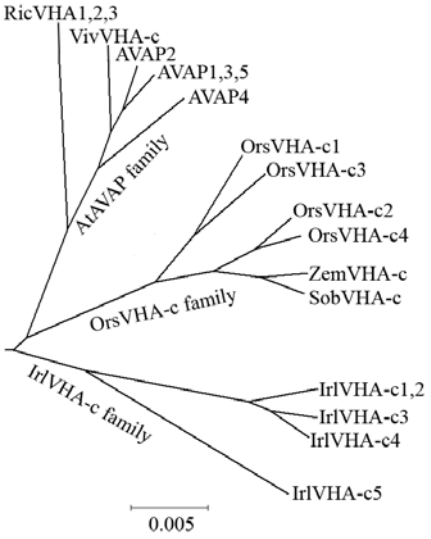

Fig. 2 Phylogeny of VHA-c proteins from several plants. ArtAVAP4 (NP177693); ArtAVAP2 (NP564098); ArtAVAP1,3,5 (NP175198, NP175603, NP179244); VivVHA-c (XP002279080); RicVHA-c1,2,3 (XP002510486); SobVHA-c (XP002441892); ZemVHA-c (NP001149195); OrsVHA-c1 (NP001047092); OrsVHA-c2 (NP001066258); OrsVHA-c3 (NP001054416); OrsVHA-c4 (NP001065855); OrsVHA-c5 (NP001045477). Sequences were aligned with the MEGA4 program using the CLUSTAW method. The scale beneath the tree indicates the distance between sequences.

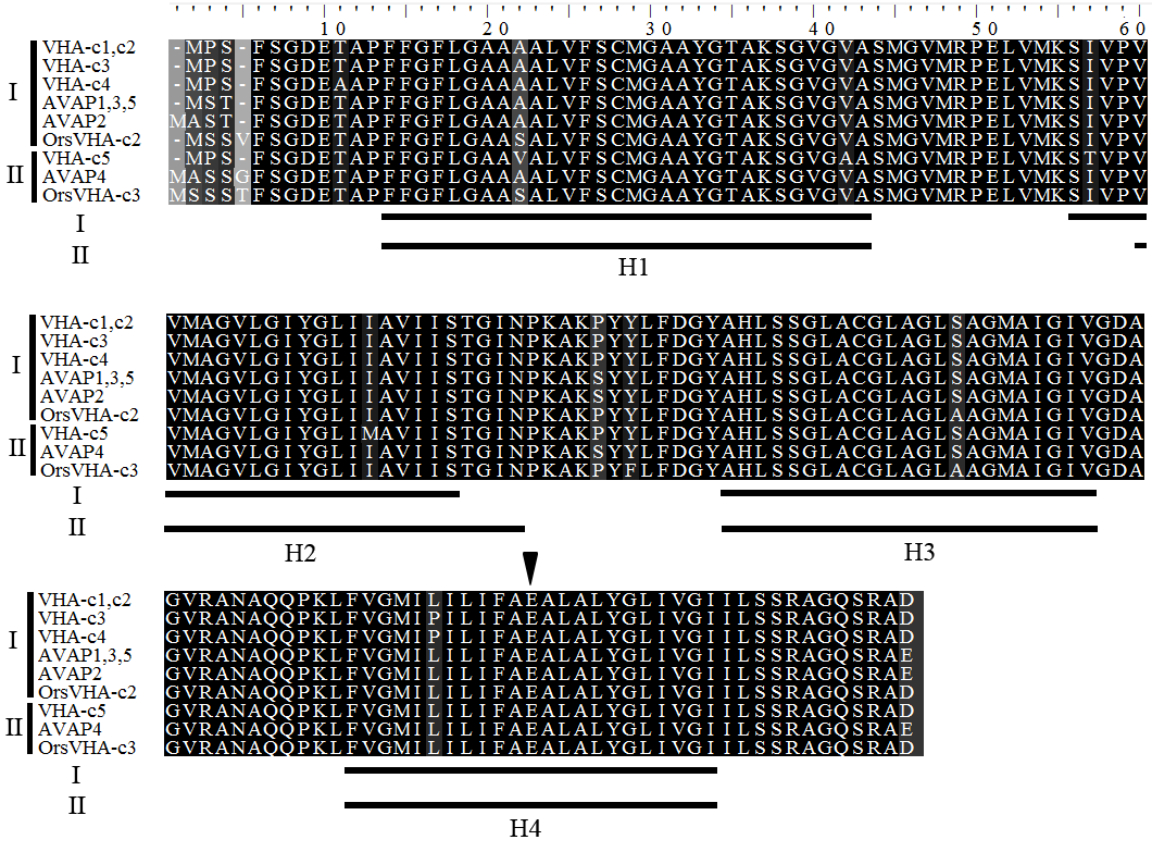

Fig. 3 Amino acid sequence alignment of *IrlVHA-c* from *Iris lactea*, AVAP from *A. thaliana* and OrsVHA-c from rice. Black and gray backgrounds indicate identical residues and similar residues, respectively. Black bars and H1~H4 indicate the putative transmembrane domains indicated by the TMHMM program. Arrows indicate the conservative proton binding sites.

Intracellular localization of the IrlVHA-c protein in yeast

The yeast transformant of pYES2-GFP was used as a control. The green fluorescence of the GFP protein alone was evenly distributed within yeast cells (Fig. 4 A, C and D), while the GFP-IrlVHA-c2−c5 fusion proteins were mainly localized in the vacuolar membrane and no green fluorescence was detected in the plasma membrane, nuclear envelopes, or free cytosol (Fig. 4 E, I, M and Q). The localization signal was consistent with the vacuolar localization signal (red fluorescence area) (Fig. 4 B, F, J,

N and R). These results indicated that *IrlVHA-c2–c5* proteins are localized in the vacuolar membrane of yeast.

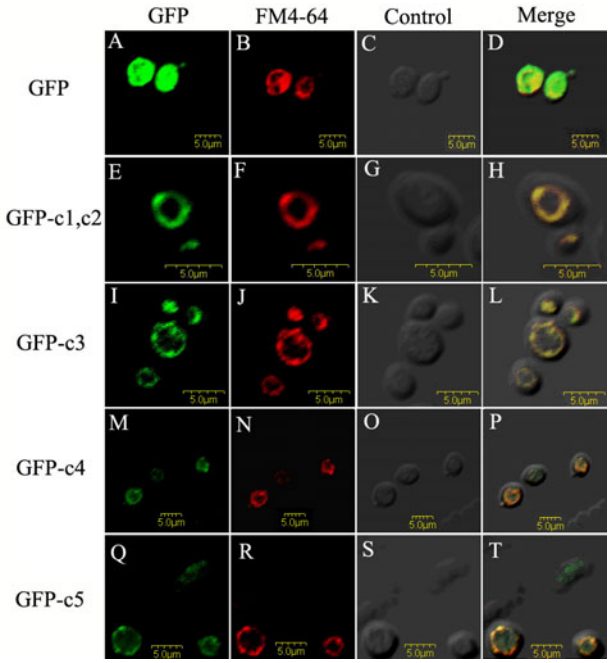

Fig. 4 GFP fusion proteins of *IrlVHA-c* are localized in the vacuolar membrane of yeast. *IrlVHA-c1~c5* were separately cloned with the GFP gene into the pYES2 vector and expressed in the yeast strain INVScI. Live cells were incubated with FM4-64 and the localization was observed with a confocal microscope. (A-D) Expression of the GFP proteins in yeast; (E-T) expression of the GFP-IrlVHA-c1~c5 proteins in yeast.

Tolerance of *IrlVHA-c* over-expressing cells to NaCl

For growth response assay, the yeast transformants of pYES2-IrlVHA-c2, pYES2-IrlVHA-c3, pYES2-IrlVHA-c4, and pYES2-IrlVHA-c5 were separately cultured in liquid SD-Uracil medium until OD_{600}≈1 and diluted by 5^{-0}, 5^{-1}, 5^{-2}, 5^{-3} and 5^{-4} fold with enriched SD-Uracil medium. Then, aliquots of each dilution were spotted onto solid yeast YPG (extract/peptone/galactose) media supplemented with different concentrations of NaCl. The yeast transformant of pYES2 empty vector was used as a control. Growth was monitored for 2−5 d at 30°C. Meanwhile, transformed yeast cells were inoculated into liquid yeast YPG media supplemented with varying concentrations of NaCl and cultured in liquid YPG medium until OD_{600}≈ 0.5. Growth was monitored for 24 h at 30°C (Fig. 5).

Yeast growth was inhibited by a medium containing 0.5 mol/L NaCl. Five transformed yeast lines were constructed. One with empty vector pYES2 was used as a control and four others were transformed with a vector containing transformants of *IrlVHA-c2–c5*. The growth of the *IrlVHA-c2–c5* transformants was the same as that of the vector transformant on YPD media. In a media containing 0.5 mol/L NaCl, growth of the *IrlVHA-c2–c5* transformants was weaker but was stronger than the growth of the pYES2 vector transformant. Meanwhile, growth patterns of the *IrlVHA-c2–c5* transformants were nearly the same. The growth patterns were identical among five transformed yeasts on YPD (extract/peptone/glucose) media. In conclusion, the *IrlVHA-c* can increase yeast tolerance to NaCl.

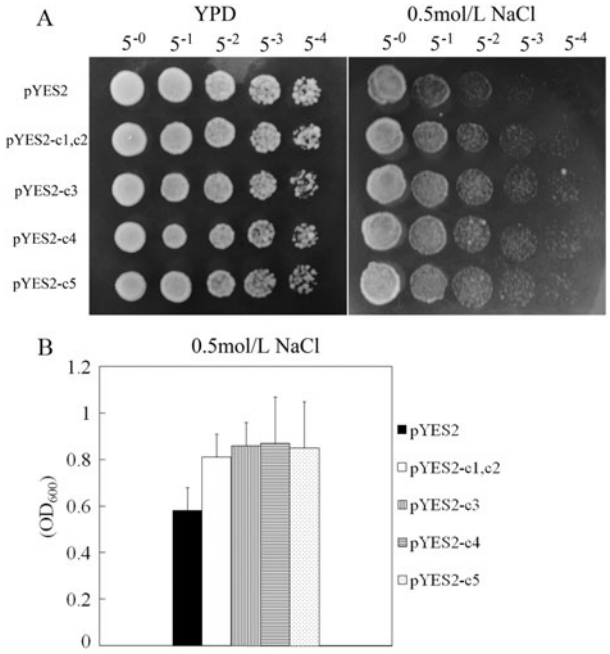

Fig. 5 Resistance of *IrlVHA-c* over-expressing cells to NaCl. (A) Yeast cells containing pYES2-IrlVHA-c and pYES2 vector were separately incubated as described in Materials and methods. Serial dilutions were spotted onto solid yeast YPG media supplemented with or without additional NaCl. Growth was monitored for 2-5 d at 30°C. (B) Growth curve of pYES2 and transformed yeast at liquid YPG media supplemented with a 0.5 mol/L NaCl. Growth was monitored for 24 h at 30°C and recorded OD_{600}.

Discussion

The pH of an intracellular compartment is a balance between active proton transport and passive proton leakage (Cipriano et al. 2008). An organellar pH may be regulated by changing passive proton conductance. Brett et al. (2005) found that intracellular Na^+/H^+ antiporters may play this role in yeast. Recent findings suggested that *PutCAX1* (gene of cation/H^+ antiporter from *Puccinellia tenuiflora*) expression affects responses to Ca^{2+} and Ba^{2+} in yeast (Liu et al. 2009). Over-expression of *GhDET3* (subunit C of V-ATPase from *Gossypium hirsuturm* L.) promoted fission and yeast cell elongation, and enhanced tolerance to high NaCl and high pH stresses (Xiao et al. 2008). Thorsten et al. (2004) found that *VHA-c* (subunits c of V-ATPase from *Mesembryanthemum crystallinum*) protein is localized at the tonoplast, the plasma membrane, and the endoplasmic membrane. We showed that *IrlVHA-c* has four isoforms which are encoded by five genes or more. We found that *IrlVHA-c* is localized on the yeast vacuole membrane and *IrlVHA-c* expression affects yeast response to NaCl. Yeast is a monoplast eukaryote. Many conserved genes from plants have similar functions in yeast and many basic transporting mechanisms are identical between plants and yeast

(Li et al. 2002). The V-ATPase subunit genes from pearl millet, lemon, *Arabidopsis*, and other species can rescue relevant mutations in yeast (Ferea and Bowman 1996; Schumacher et al. 1999; Keren et al. 2000; Wricha et al. 2005). Therefore, yeast is a useful organism for functional analysis of genes from plants (Xiao et al. 2008). In this research, we used the yeast system to express the *IrlVHA-c* genes from plants and analyze their functions.

Subunit c within the complex V-ATPase plays a central role. Absence of this subunit will cause other subunits of V_0 to be unassembled (Nelson and Klinsky 1996). The binding between the subunit c of V_0 and two subunits of V_1, namely subunits B and E, will cause subdomains V_0 and V_1 to unite (Inoue and Forgac 2005). The proteolipid subunits form a ring containing a single copy of subunit c′ and c″ and 4-5 copies of subunit c (Arai et al. 1988; Powell et al. 2000). Subunit c has multiple isoforms and each isoform shows different expression patterns, suggesting that each might have different regulatory functions in response to developmental and environmental cues (Tyagi et al. 2006). In future, we plan additional study to learn whether the hypothesized functional differences between the five isoforms prove valid.

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